Fluctuating asymmetry and immune function in a field cricket

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Recently, fluctuating asymmetry (FA) of morphological traits has attracted great attention as a short-cut measure of individual quality. Whereas there is some evidence that FA of sexual ornaments is negatively associated with immune function, studies concerning FA and immune function in non-ornamental traits are absent. Here, we tested whether FA of three non-ornamental traits in hind limbs is related to male immune function in a population of the Mediterranean field cricket, *Gryllus bimaculatus*. As different measures of male immune function, we used encapsulation rate and lytic activity. We found that a composite measure of FA (cFA) was negatively related to encapsulation rate. However, lytic activity was not related to cFA, but there was a tendency that males with higher body mass had higher lytic activity than males with lower body mass. Our results suggest that FA in non-ornamental traits indicates male immunocompetence in *G. bimaculatus*.

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Fluctuating asymmetry (FA) of a bilateral trait is defined as subtle, random deviations from a genetically controlled trajectory towards an optimal phenotype, i.e. perfect symmetry (Van Valen 1962, Palmer and Strobeck 1986). FA has recently been proposed to indicate individual quality (reviewed by Møller and Swaddle 1997, Møller and Thornhill 1998, Thornhill et al. 1999, but see Palmer 1999, Simmons et al. 1999). This proposal is based on the assumption that FA is a sensitive measure of environmental and genetic stress, and has lead to the prediction that FA should covary negatively with condition (Møller 1990, Møller and Pomiankowski 1993, Watson and Thornhill 1994). A negative relationship between FA and immune function may arise for at least three different reasons (Møller 1996). First, poor condition may lead to both large FAs and high prevalence of parasite infections (Table 1 in Møller 1996). Second, if competitive ability depends on body condition, developmentally unstable individuals will, all else being equal, more often be restricted to poor environments with elevated risk of encountering parasites. Third, parasites may exploit the limited resources of their hosts thereby disrupting the optimal ontogeny. This can be the direct cause of large FAs. Therefore, through the mediating mechanism of condition-dependence, immune function may be negatively related to FA at the population level (van Noordwijk and de Jong 1986, Folstad and Karter 1992, Møller and Saino 1994).

There is some evidence that FA in sexual ornaments is negatively associated with immune function. For example, Lagesen and Folstad (1998) found that male reindeers with more symmetrical antlers had better immune function than less symmetrical males. Likewise, Rantala et al. (2000) found that in damselfly, *Calopteryx splendens,* males with more symmetrical wing spots had better immune function than less symmetrical males. However, studies concerning FA and immune function in non-ornamental traits are absent to our knowledge.

Insect immune system includes cellular and humoral components which interact with each other in defending against parasitoids, viruses, bacteria and fungi (Washburn et al. 1996, Gillespie et al. 1997, Brey and Hultmark 1998). One of the most informative ways to assay immunocompetence (i.e. an animal's ability to mount an effective immune defence) is to measure the magnitude of the encapsulation response to a novel and standardized antigen such as a nylon monofilament (Köning and Schmid-Hempel 1995, Rantala et al. 2000, 2002, 2003a, b, Ryder and Siva-Jothy 2000, 2001, Siva-Jothy 2000, Koskimäki et al. 2003, Rantala and Kortet 2003, 2004, Vainio et al. 2004). In the encapsulation response, circulating cells in the haemocoel recognize multicellular pathogens as foreign and form hard capsules around them. Capsules forming are covered by the black pigment, melanin, which is the result of the prophenoloxidase (PPO) cascade. The encapsulated organism faces several killing factors, e.g. asphyxiation (Fisher 1963) and the production of necrotising compounds (Nappi et al. 1995). The humoral immune response targets microbial pathogens and is characterized by the rapid production of a number of antimicrobial peptides as well as the activation of the PPO cascade leading to melanization (Brey and Hultmark 1998).

The Mediterranean field cricket, *Gryllus bimaculatus* De Geer, is widely distributed in southern Europe, where it frequently occurs in high densities. Females of *G. bimaculatus* are sensitive to variation in the syllable rate of male calls (Doherty 1985, Schildberger 1985) preferring male calls with high syllable rates (Shuvalov and Popov 1973, Simmons 1988). There is also evidence that male body size influences male mating success (Simmons 1986, Bateman et al. 2001). Recently, it has been shown that females of *G. bimaculatus* prefer courtship song of males with high encapsulation response over males with low encapsulation response (Rantala and Kortet 2003), and males with better immunocompetence are more successful in male–male competition (Rantala and Kortet 2004). The aim of this study was to test whether FA in three non-ornamental traits is associated with male immune function in *G. bimaculatus*. As different estimates of male immune function, we used the encapsulation rate against a novel antigen and the haemolymph concentration of an antibacterial enzyme, lysozyme.

Material and methods

Insects

Males of *G. bimaculatus* were a random first generation sample of specimens collected from Costa del Sol, southern Spain (10 females and 25 males). They were mass-reared in the laboratory, and experimental crickets were derived from a bulk laboratory stock as small nymphs. Sexes were physically (but not acoustically) isolated from other individuals, and thus they were all virgins. They were individually maintained in covered plastic containers (1 l) at $+29°C \pm 1$ under a 12:12 h light/dark photoperiod with ad libitum food and water. All males were eight days old at the beginning of the experiments. Before the immunological assays, we weighed the fresh body mass of crickets to the nearest 0.01 g. The mean body mass ($\pm$SE) was 0.70 g$\pm$0.02 (n$=$69).

Encapsulation response assay

To measure the encapsulation rate, males were chilled on ice for 20 min after which a two millimetre long piece of nylon monofilament (Ø$=$0.18 mm) was inserted into the cricket's haemocoel through a puncture in the pleural membrane between the second and third sternite. The males' immune system was allowed to react to this object for five hours, while keeping crickets individually in plastic vials at constant room temperature ($+28°C\pm1$). The implant was then removed and dried. The removed monofilament was photographed under a light microscope with a digital video recorder from three different angles. The pictures were then analysed using the Image Pro-program. The encapsulation rate was analysed as grey values of reflecting light from implants. As a measure of the encapsulation rate, we used the average grey value of three video pictures. We transformed the data such that the darkest grey values correspond to the highest encapsulation rates. We did this transformation by subtracting observed grey values from the control grey value (clear implant). The repeatability (R) of this method has been shown to be very high (Rantala et al. 2002).

Lysozyme assay

After the encapsulation rate measurements, we collected 10 μl of haemolymph from each male from the puncture in their abdomen. We assayed lysozyme activity of haemolymph against *Micrococcus lysodeikticus* turbidometrically using methods similar to those used in Rantala and Kortet (2003). We mixed 200 μl of 0.35 mg ml^{-1} freeze-dried *M. lysodeikticus* buffered (pH 6.4) solution with 50 μl 1:4 buffered haemolymph (pH 6.4) in a plastic multicuvette (Labsystems cliniplate). The optical density of the mixture at 492 nm was then measured at $+20°C$ in one minute intervals for 30 min with a plate reader (Multiskan Plus, Labsystems, Finland). The lytic activity was expressed as the change in optical density.

Measurements of fluctuating asymmetry (FA)

To prepare crickets for FA measurements, we killed crickets by freezing them at $-20°C$. One of the authors (J. J. Ahtiainen) made all the FA measurements to eliminate any between-observer variation in measurements (Hubert and Alexander 1995). He cut off the hind limbs from the abdomen under a binocular microscope. Then, he photographed the hind limbs under a light microscope with a digital video recorder. The lengths of the femur, tibia and tarsus were measured to the nearest 0.001 mm from both hind limbs using the Image Pro® Plus–software (ver. 4.1). The repeatabilities of asymmetries of the femur, tibia, and tarsus were calculated by repeating the measurements of each trait. The repetition procedure was as follows: first, the left-hand side segments (L) were measured once, followed by the measurements of the right-hand side segments (R) in a random order without reference to the measurements of the left-hand side segments. Re-measuring the same individuals was done with exactly the same procedure, and in a random order without reference to the first measurements (Palmer 1994). FA measurements were made blindly with respect to immunological measurements. Asymmetry of each trait was then defined as the mean length difference between left- and right-hand side segments. For all traits, the measurement repeatability for unsigned asymmetry was high (femur: $R = 0.77$, $F_{68,138} = 7.49$, $p < 0.001$; tibia $R = 0.98$, $F_{67,136} = 97.22$, $p < 0.001$; tarsus: $R = 0.99$, $F_{63,64} = 152.42$, $p < 0.001$).

FA analysis

It has been shown that the admixture of FA with antisymmetry results in leptokurtic or platykurtic distributions with zero mean, while the admixture of FA with directional asymmetry skews the distribution of the signed asymmetry (Palmer and Strobeck 1992, Rowe et al. 1997, Van Dongen 1998). As antisymmetry and directional asymmetry can have a genetic basis, those asymmetries do not necessarily reflect developmental stability (Palmer and Strobeck 1992, Palmer 1994, but see Graham et al. 1993). It is possible to separate FA and directional asymmetry by a two-way mixed model ANOVA (Palmer and Strobeck 1986). Whereas FA and antisymmetry cannot be separated statistically with high power, they can be distinguished reliably through inspection of the scatter plot between trait size and unsigned FA (Rowe et al. 1997).

In the Results, we used a composite measure of FA (cFA). We tested several composite measures of FA, and nonparametric, ranked, summed FA values provided the greatest power in detecting FA-fitness relations (Weatherhead et al. 1999, Leung et al. 2000). We computed the composite asymmetry scores (cFA_i) as the sum of ranked FA values across traits (j) for each individual (i).

$$cFA_i = \Sigma(j = 1 \text{ to } k) \text{ ranked } |FA_{ij}|$$

Because of the few missing values, sample sizes are not equal in the Results. Because traits measured in this study were not normally distributed, we used non-parametric Spearman's correlation coefficients.

Results

In order to test whether there was any true FA separable from directional asymmetry and measurement error, we tested asymmetries for each trait by the two-way mixed model ANOVA (Palmer and Strobeck 1986). The mean signed FA values for each trait did not differ from zero, indicating that there was no directional asymmetry in the FA distributions of femur, tibia or tarsus (significance test for directional asymmetry: femur $F_{1,68} = 0.482$, $p > 0.05$; tibia $F_{1,67} = 0.131$, $p > 0.05$; tarsus $F_{1,63} = 3.737$, $p > 0.05$). Visual inspection of signed asymmetry distributions indicated that all traits fulfilled the patterns expected for FA (i.e. a normal or kurtosis distribution with a mean of zero). In all traits, the true between-sides variance, i.e. the FA variance, differed significantly from the measurement error variance (femur: $F_{68,138} = 5.282$, $p = 0.025$, measurement error variance $\sigma_m^2 = 0.003$; tibia: $F_{67,136} = 5.445$, $p = 0.023$, $\sigma_m^2 = 0.002$; tarsus: $F_{63,128} = 5.163$, $p = 0.026$, $\sigma_m^2 = 0.003$), indicating that FA can be separated out from measurement error. A careful inspection of the relationship between trait size and unsigned FA indicated that there was no antisymmetry in the FA distributions of femur, tibia or tarsus. Mean relative unsigned FA (controlling for trait size) of the femur was 0.013 ± 0.001 mm, of the tarsus was 0.026 ± 0.006 mm, and of the tibia was 0.085 ± 0.020 mm ($\pm SE$).

There was a significant moderate negative correlation between cFA (composite FA) and encapsulation rate (Spearman's $r_s = -0.318$, $n = 56$, $p = 0.017$; Fig. 1). However, there was no significant correlation between cFA and lytic activity (Spearman's $r_s = -0.109$, $n = 61$, $p = 0.404$). cFA did not correlate significantly with body mass (Spearman's $r_s = 0.129$, $p = 0.317$, $n = 62$). Whereas encapsulation rate did not correlate significantly with body mass (Spearman's $r_s = -0.099$, $n = 64$, $p = 0.435$), there was a positive tendency between lytic activity and body mass (Spearman's $r_s = 0.220$, $n = 69$, $p = 0.069$).

Discussion

The main result of this study was that, in *Gryllus bimaculatus* males, encapsulation rate is negatively related to hind limb FA at the population level. This suggests that FA in non-ornamental traits might be used as a measure of individual quality in *G. bimaculatus*, provided that the number of traits and within-trait
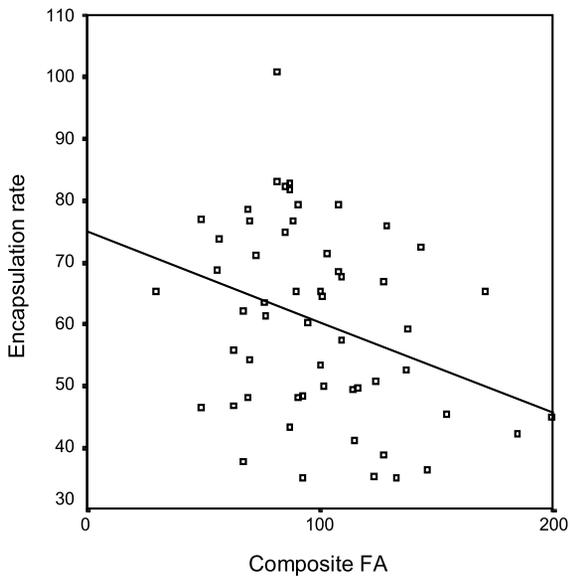
Fig. 1. Encapsulation rate and composite FA (cFA).

repeats as well as sample sizes are large enough to attain high reliability (Leung et al. 2000, Ahtiainen et al. 2003). Symmetry in hind limbs might be a biomechanical advantage maintaining stability and balance in a variety of postures and motion (Sneddon and Swaddle 1999). This might be critical for good performance in predation, which might in turn influence immune function through the intake of essential nutrients (e.g. carotenoids; Folstad et al. 1994). Symmetry in hind limbs might also influence male sexual performance. Recently, Mallard and Barnard (2004) have shown that FA is greatest and reproductive performance is weakest when *G. bimaculatus* males are reared on impoverished food. There is also some evidence that immune function in insects is condition-dependent. For example, in *Anopheles gambiae* (Diptera: Culicidae) larval nutrition affects the ability of adults to respond to synthetic immune challenge (Suwanchaichinda and Paskewitz 1998). Rantala et al. (2003a) have found that male nutritional condition affects phenoloxidase (PO) activity but not encapsulation rate. However, encapsulation rate might be partly condition-dependent, as it mainly measures melanization rate, melanin being an end product of the PO cascade. Therefore, condition-dependence might be a mediating mechanism behind the relationship between FA and encapsulation rate in *G. bimaculatus*, only males in good condition being able to produce both symmetrical hind limbs and effective encapsulation responses.

Only one study has examined the relationship between FA and immune function in invertebrates. In that study, FA was measured from an ornament of *Calopteryx*

splendens, wing spots, and the result was that males with less symmetrical wing spots had lower encapsulation rates than males with more symmetrical wing spots (Rantala et al. 2000). In this study, we did not find any correlation between FA and lytic activity. This might be, at least partly, owing to small sample sizes, as statistical power increases with sample size.

We did not address the causal factors behind the relationship between hind limb FA and encapsulation rate. However, we suggest that one explanation for the observed relationship is more plausible than the others. As environmental conditions were similar for all specimens in our study, the observed relationship might be due to differences in innate male competitive ability. This means that, even though there were no other males competing for resources in our rearing procedure, males might be genetically programmed to optimize their development into maximal growth rate. This could lead to differences in condition at the age of eight days influencing both hind limb FA and encapsulation response. The support for this argument comes from the rearing conditions. We raised crickets individually in optimal conditions with ad libitum food and water, thus minimizing environmental effects. Moreover, we did not detect any parasitoids or diseases in the experimental animals.

Our estimate of $r_s = -0.32$ is consistent with the weighted mean effect size for FA in ordinary traits versus male quality, which was $r = -0.29$ ($p < 0.05$, total estimates = 37 studies; Møller and Thornhill 1998). This extensive meta-analysis also showed that there are greater negative correlations between FA and male quality, when secondary sexual traits rather than ordinary traits are studied. This is because secondary sexual traits are the focus of sexual selection showing greater levels of FA (Møller and Thornhill 1998). Therefore, it would be surprising if the relationship between FA in non-ornamental traits and immune function were high. Accordingly, recent studies with large sample sizes show only weak relationships between FA in ordinary traits and male quality (Ahtiainen et al. 2003).

Simmons (1995) has found that *Gryllus campestris* males with higher body mass were more symmetrical and older than males with smaller body mass. We did not find any correlation between body mass and FA in *G. bimaculatus* males at the age of eight days. Moreover, we did not find any correlation between body mass and encapsulation rate. However, there was a tendency for males with higher body mass to have higher lytic activity than males with lower body mass. Several studies in the Mediterranean field cricket have shown that male body size influences male mating success (Simmons 1986, Bateman et al. 2001). Therefore, future studies should examine with larger sample sizes whether body mass is also indicative of male immune function.

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